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(71) Applicant: **SOCIETE DES PRODUITS NESTLE [NESTLE PRODUCTS COMPANY] S.A.**
P.O. Box 353
CH-1800 Vevey (CH)

CH-1015 Lausanne (CH)

Inventor: **Meister, Niklaus**
Möschbergweg 20
CH-3506 Grosshoechstetten (CH)

Inventor: **Renken, Albert**
EPFL,
Institut de Génie Chimique CHB
[Chemical Engineering Institute]
CH-1015 Lausanne (CH)

Inventor: **Wood, Robert Dustan**
Av. d'Echallens 63
CH-1004 Lausanne (CH)

Inventor: **Woupeyi, Alfred**
Rue Haldimand 24B
CH-1400 Yverdon (CH)

(72) Inventor: **Baensch, Johannes**

Rte du Port 28

CH-1009 Pully (CH)

Inventor: **Margot, Antoine**

EPFL,

Institut de Génie Chimique CHB

(74) Agent: **Wavre, Claude-Alain**

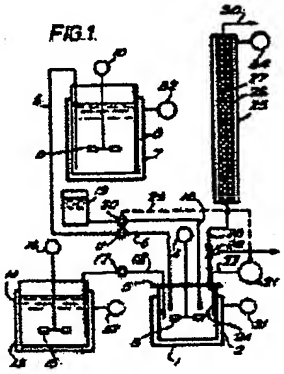
et al.

55, avenue Nestlé

CH-1800 Vevey (CH)

(54) **Enzymatic Hydrolysis of Proteins.**

(57) Process and device for enzymatic hydrolysis of proteins, in which are mixed a proteolytic enzyme and a protein substrate; a first stage of hydrolysis is carried out in a tank while being stirred, and a second stage of hydrolysis is carried out in a tube that is equipped with static mixer elements.



EP 0 566 877 A2

This invention has as its object a process for enzymatic hydrolysis of proteins and a device for implementing the process.

Various processes for enzymatic hydrolysis of proteins that are distinguished by the selection of substrate, enzyme, degree of hydrolysis and/or desired peptide profile, for example, are known. When, for reasons of assimilation of the hydrolyzate by intestinal mucous membranes, for example, a relatively well-defined peptide profile, in particular a narrow oligopeptide profile, is desired, the known hydrolysis processes generally comprise at least one stage for filtering or sieving the hydrolyzate.

Thus, EP 226221, for example, describes a process for preparation of hypoallergenic peptides of a molecular weight of between 2000 and 6000 per one or more stages of enzymatic hydrolysis of proteins each carried out intermittently in a fermentation tank and each ended by an ultrafiltration stage.

US 4212889 describes a process for solubilization of fish proteins, in which a mixture of fish skin and enzyme pulp is continuously run through an installation that comprises several hydrolysis tanks that are connected in series.

This invention has as its object proposing a hydrolysis process that, while preferably being carried out continuously, made it possible to equal, and even to improve, the efficiency of a batch hydrolysis in a tank and that made it possible to obtain a protein hydrolyzate that exhibits a degree of hydrolysis and/or a peptide spectrum that is well-defined and reproducible.

For this purpose, the process for enzymatic hydrolysis of proteins according to this invention, in which a protein substrate is subjected to hydrolysis with a proteolytic

enzyme, comprises a first stage of enzymatic hydrolysis in a tank while being stirred and a second stage of enzymatic hydrolysis in a tube. This process is preferably carried out continuously, and the second stage of enzymatic hydrolysis in a tube that is equipped with static mixer elements is carried out.

Likewise, the device for the implementation of this process comprises a double-jacket hydrolysis tank with a stirring mechanism that is connected upstream to a substrate-metering device and to an enzyme-metering device and connected downstream to at least one hydrolysis tube. Said tube is preferably equipped with static mixer elements.

It was noted that it was thus possible to produce, preferably continuously, with good efficiency and good reproducibility, a protein hydrolyzate that exhibits a degree of hydrolysis and/or a peptide spectrum that are well defined.

Thanks to this process and this device, it is possible in particular to work with a tank of relatively small size that can be filled completely, without leaving any head room, and with a tube of relatively large size. It is therefore possible to carry out, preferably continously, a relatively short first stage, in other words a stage of launching hydrolysis in a relatively small tank, and a relatively long second stage, in other words a stage for finishing hydrolysis in a tube that exhibits a relatively large volume. It thus is possible to control precisely and simply, for example via volumetric pumps, the reaction time, in other words the dwell time of the substrate in the total volume represented by the sum of the volume of the tank and the volume of the tube.

If, for comparison, it is desired to carry out hydrolysis in a single large hydrolysis tank, the dwell time of an elementary volume of hydrolyzate cannot be defined

specifically. This is true for a batch hydrolysis, whereby the time that is necessary for establishing given conditions of pH and/or temperature, and even for emptying the tank, for example, is significant. This is all the more true, however, for a continuous hydrolysis, for which it is possible to define only an average dwell time. Even in a process such as that of US 4212889 cited above, the dwell time can hardly be defined more specifically.

In contrast, it turns out that with the process and the device according to this invention, the dwell time of an elementary volume of hydrolyzate can be defined in a remarkably specific manner, whereby the stream of hydrolyzate in the hydrolysis tube, which is preferably equipped with static mixer elements, can have a very flat front.

In this disclosure, a degree of hydrolysis is defined by the non-protein amount of nitrogen (NPN) that is determined as the percentage of total nitrogen that cannot be precipitated with trichloroacetic acid at 13%.

The nitrogen contents are determined by the Kjeldahl method.

The contents of amine nitrogen (free alpha-NH_2) are determined by reaction to ninhydrin after alkaline hydrolysis.

The tests for releasing tritium-labeled exogenic serotonin (serotonin-^3H) are done on normal mastocytes of the peritoneal cavity of the rat according to the method that is described by R. Fritsché and M. Bonzon in Int Arch Allerg Immunol 93, 289-293 (1990).

The ELISA inhibition tests are done with specific rabbit antibodies of beta-lactoglobulin (BLG), bovine serum albumin (BSA) and casein (CAS). The sensitivity of the method, in other words the detection concentration limit, is 20 ng/ml.

The high-performance liquid chromatography analyses (HPLC tests, peptide profiles) are carried out under non-denaturing conditions on a TSK-G2000-SW-type silica-based gel of the Toyo Soda Company, whose fractionation range extends from 500 to 50,000 Dalton, in a BIOSIL SEC-125 column of the BIORAD Company. The results are expressed in % of surface area distribution of the peaks read at 220 nm in 0.1 M phosphate solution + 0.4 M NaCl at pH 6.80.

The analyses by polyacrylamide-gel zone electrophoresis (SDS-PAGE tests) are carried out according to the method that is described by Laemmli in Nature 227, 680 ff (1970).

The lysine blockage is determined by HPLC and expressed in % of blocked lysine relative to the total lysine of the hydrolyzate.

By the expression "static mixer elements," it is possible to include braces or corrugated strips made of metal or of plastic that intersect or overlap and that subdivide the space that is delimited by said tube into a number of channels that intersect as they move forward into the axis of the tube. Such elements are marketed by the Sulzer A. G. Company, CH-8401 Winterthur, under the name SMV, SMX or SMXL, for example.

Finally, in this disclosure, it is necessary to understand that said tubes that are equipped with static mixer elements are systematically equipped with a double jacket, even when this is not specified.

To implement this process, it is possible to use as a protein substrate any alimentary raw material that is high in proteins, such as flours or semolinas of grains or oleaginous oilcakes, yeasts or alimentary bacteria, animal skin or ground fish, or milk or

milk derivatives, in the form of particles of aqueous suspension or aqueous suspension, for example.

Said protein substrate is preferably a lactose substrate that contains proteins of lactoserum, in particular a mild cheese-dairy lactoserum or an acidic cheese-making lactoserum, as is or in demineralized form or delactosed form, in liquid form or in reconstituted form.

The enzyme is also preferably selected from the group that is formed by trypsin, chymotrypsin, pancreatin, bacterial proteases, fungal proteases and mixtures thereof.

It is possible to mix the proteolytic enzyme and the substrate at a rate of an amount of enzyme that exhibits an activity of 0.1-12 Anson units (AU) per 100 g of dry substrate material.

The first stage of hydrolysis is preferably carried out for 10-60 minutes while adjusting the pH and the temperature to values that are favorable to the activity of the enzyme, and the second stage of hydrolysis is preferably carried out for 1-8 hours by adjusting the temperature to a value that is greater than or equal to, in particular 0-10°C, the temperature of the first stage.

It is possible to provide intermediate or additional stages, in particular a stage of preliminary mixing, preferably in a tube that is equipped with static mixer elements; a heat denaturation stage, before, in the middle of, or after the first stage of hydrolysis, in particular with a heat exchanger or a tube that is equipped with static mixer elements; one or more enzyme deactivation stages, and in particular after the second hydrolysis stage, in particular with a heat exchanger and/or a vapor injection device and/or a tube that is equipped with static mixer elements; and/or a stage of cooling, in particular after a

denaturation stage, in particular with a heat exchanger or preferably in a tube that is equipped with static mixer elements, for example.

It is possible to deactivate the enzyme in one, or preferably two stages, a first stage corresponding more specifically to an enzyme auto-digestion and a second stage that corresponds more specifically to sterilization.

It is also possible to subdivide said first hydrolysis stage in a tank into at least two portions that are carried out in at least two tanks that are connected in series. Likewise, it is possible to subdivide said second hydrolysis stage in a tube into at least two portions that are carried out in at least two tubes that are connected in series. In the latter case, it is possible to carry out an adjustment of pH and/or to add the enzyme between two successive tubes.

For the adjustment or adjustments of pH, a suitable reagent is preferably used, either an alkaline reagent, such as KOH, NaOH or $Ca(OH)_2$, or an acidic reagent, such as HCl or HPO_4, for example.

In a preferred embodiment of this process, a bacterial alkaline protease, in particular the one that is produced by Bacillus licheniformis and marketed by the Novo Company under the name "alcalase," in particular "alcalase 0.6 L" or alcalase 2.4 L", for example, is selected as an enzyme.

It is noted that with this preferred embodiment, it was possible to obtain a hydrolyzate that exhibits a particularly high NPN and a particularly reduced allergenicity.

To do this, it is possible to carry out said first hydrolysis stage at a pH of 7.0-10.0 at 50-80°C, preferably 63-73°C, and the second hydrolysis stage at a pH of 6.5-8.0 at 55-80°C, preferably 65-73°C. It is possible to provide, either before the first hydrolysis

stage, or between said two hydrolysis stages, a stage of heat denaturation at 80-120°C, preferably at 85-95°C, for 30 seconds to 10 minutes, preferably for 4-6 minutes. It is then possible to deactivate the enzyme by an auto-digestion stage at 70-110°C, preferably 85-90°C, for 10 seconds to 20 minutes, preferably for 2-8 minutes, followed by a sterilization stage at 110-150°C, preferably 120-130°C, for 5 seconds to 5 minutes, preferably for 30 seconds to 2 minutes.

In another preferred embodiment of this process, a combination that comprises, on the one hand, a bacterial alkaline protease, in particular the one that is produced by Bacilus licheniformis and is marketed by the Novo Company under the name "alcalase," in particular "alcalase 0.6 L" or "alcalase 2.4 L," and, on the other hand, a pancreatic enzyme, in particular trypsin, for example, is selected as an enzyme.

In this other preferred embodiment, it is possible to subject two substrates separately, in particular two lactose substrates, each to a separate hydrolysis, with one of these two enzymes, according to this process, up to a common stage, preferably up to a common sterilization stage following two separate stages of auto-digestion of two different enzymes. It is also possible to subject the same substrate successively to the action of one and then the other of these two enzymes. It was actually noted that a hydrolysis product of the lactoserum proteins, for example obtained by this combination, can have particularly long shelf lives.

For the implementation of this process with a pancreatic enzyme, in particular trypsin, for example, in particular within the framework of the combination above, it is possible advantageously to use the conditions of pH and temperature that are described in EP 322589, whose content is integrated in this description by reference.

The device for the implementation of the process according to this invention therefore comprises a double-jacket hydrolysis tank with a stirring mechanism that is connected upstream to the substrate-metering device and to an enzyme-metering device, and connected downstream to at least one hydrolysis tube that is equipped with static mixer elements.

In this device, said tube can be placed vertically, whereby its lower end is connected to said tank and its upper end empties into a discharge hose. It may also be placed horizontally or in any other position. It preferably exhibits a length of more than 4x its diameter.

Said substrate-metering devices and enzyme-metering devices also preferably each have a feed container that is connected to said hydrolysis tank via a volumetric pump.

The device can also comprise a device that meters reagent and that has a feed container that is connected to the hydrolysis tank via a volumetric pump that is controlled by a pH meter.

The device can also comprise several tanks that are connected in series instead of a single tank, in particular two tanks of which one can be used in prehydrolysis.

The device can also comprise several hydrolysis tubes that are equipped with static mixer elements, connected in series downstream from the tank via connecting hoses that can be connected upstream to the enzyme-metering device and the reagent-metering device.

It is also possible to provide a tube that is equipped with static mixer elements between said devices that meter enzyme, that meter substrate and/or that meter reagent, and said tank, even between two successive tanks if the device comprises several tanks.

The device for the implementation of the process according to this invention is described below in reference to the accompanying drawing that illustrates, by way of example, three embodiments thereof. In this drawing,

- Figure 1 diagrammatically shows a first embodiment of the device, comprising a tank and a tube that is equipped with static mixer elements,
- Figure 2 diagrammatically shows a second embodiment of the device, comprising a tank and several hydrolysis tubes that are equipped with static mixer elements, and
- Figure 3 diagrammatically shows a third embodiment of the device, comprising two tanks and several hydrolysis tubes equipped with static mixer elements.

As can be seen in Figure 1, this device comprises a hydrolysis tank 1 with a double jacket 2 with a stirring mechanism 3 that is driven by a motor 4. This tank is closed in an airtight manner by a cover 5 through which run various hoses and the axis of stirring mechanism 3.

Hydrolysis tank 1 is connected upstream via a hose 6 to a substrate-metering device 7-11, via a hose 12 to an enzyme-metering device 13-17, and via a hose 18 to a reagent-metering device 19-24.

The substrate-metering device comprises a substrate feed container 7 with a double jacket 8 and stirring mechanism 9 that is driven by a motor 10. Container 7 is connected to hydrolysis tank 1 via volumetric pump 11 that is connected to hose 6.

The enzyme-metering device has an enzyme feed container 13 with a double jacket 14 and stirring mechanism 15 that is driven by a motor 16. Container 13 is connected to hydrolysis tank 1 via volumetric pump 17 that is connected to hose 12.

The reagent-metering device comprises a reagent feed container 19 that is connected to hydrolysis tank 1 via a volumetric pump 20 that is connected to hose 18. This volumetric pump 18 is controlled by a pH-meter 21 whose measuring electrode 24 is immersed in tank 1 through cover 5 and that is connected electrically (dashed line 23) to an electronic control device of pump 20, not shown.

Hydrolysis tank 1 is connected downstream to a hydrolysis tube 25 with a double jacket 26 that is equipped with static mixer elements 27 that consist of crosses made of metal or plastic that overlap. Tank 1 is connected to tube 25 via a hose to which is connected a three-way valve 29 that is intended to allow hydrolyzate sampling in the tank.

Tube 25 is placed vertically, whereby its lower end is connected to tank 1 and its upper end empties into discharge hose 30.

The temperature of a fluid circulating in each of the double jackets is regulated by a device that is shown symbolically by 31 for tank 1, 32 for container 7, 33 for container 13, and 34 for tube 25.

In Figure 2, the elements of this second embodiment of the device that correspond to the elements of the first embodiment according to Figure 1 are designated there by the same reference numbers.

In this second embodiment, the device comprises several hydrolysis tubes 25, 35, 36 that are equipped with static mixer elements 27, 37 and 38, and are connected in series

downstream from tank 1 via connecting hoses 39, 40 that are connected upstream to enzyme feed container 13 via hoses 41, 42 that join with hose 12 to which is connected volumetric pump 17.

These connecting hoses 39, 40 are also connected upstream to reagent feed container 19 via hoses 43, 44 that join with hose 18 to which is connected volumetric pump 20.

In this second embodiment of this device, a mixing tube 45 equipped with static mixer elements between the devices for metering enzyme, for metering substrate and for metering reagent and tank 1 is also provided.

The different enzyme, substrate and reagent feed containers are connected to this tube 45 via hoses 46, 47, 48 to which are respectively connected a volumetric pump 49 and volumetric pumps 11 and 20.

In Figure 3, the elements of this third embodiment of the device that correspond to the elements of the first two embodiments according to Figures 1 and 2 are also designated there by the same reference numbers.

In this third embodiment, the device comprises several hydrolysis tubes 25, 35, 36, 50 that are equipped with static mixer elements and connected in series downstream from hydrolysis tank 1. The latter is connected upstream to a second tank, in this case, a prehydrolysis tank 51, via a denaturation tube 52 that is equipped with static mixer elements.

In this embodiment, prehydrolysis tank 51 is connected upstream to substrate feed container 7 and reagent feed container 19, whereas enzyme feed container 13 is

connected downstream, via respective hoses 56, 12 and 41, to prehydrolysis tank 51, hydrolysis tank 1 and connecting hose 39 of hydrolysis tubes 25 and 35.

In this embodiment, deactivation tubes 53, 54 and a cooling tube 55 that are equipped with static mixer elements and are connected in series downstream from last hydrolysis tube 50 are also provided.

The examples below are presented by way of illustration of the process according to this invention. The percentages and proportions are indicated in terms of weight.

Example 1

This process is implemented with a device that is similar to the one that is described in reference to Figure 1, in which the hydrolysis tank has a volume of 30 l and the hydrolysis tube that is equipped with static mixer elements has a volume of 180 l for a height of 3 m.

A concentrate of partially demineralized lactoserum proteins that has a content of dry material of 20% and respective contents, in % on dry material, of about 23% proteins, 1.9% fatty substance, 73% lactose and 1.3% ashes is used as a substrate.

Porcine trypsin that has an activity of 3 AU/g, at a rate of 1 g of enzyme per 100 g of dry material of the substrate, or 3 AU per 100 g of dry material of the substrate, is used as an enzyme.

2N KOH is used as a reagent.

First, the tank is filled with substrate, the enzyme is mixed therein, and the batch hydrolysis process is started at pH 7.3 at 60°C for 15 minutes, and then the hydrolyzed substrate has an NPN of 40%.

The process is then continued in steady state at a flow rate such that the mean dwell time of the substrate in the tank is 30 minutes and the dwell time of the hydrolyzate in the tube is 3 hours. A temperature of 60°C and a pH of 7.3 are maintained in the tank. A temperature of 60°C is maintained in the tube, and the pH is allowed to vary, such that it spontaneously decreases from about 7.3 at the inlet to about 6.9 at the outlet.

The hydrolyzate has an NPN of 65% at the outlet of the tube.

If, for comparison, a batch hydrolysis of the same substrate is carried out with the same enzyme in the same enzyme-substrate ratio, in a 200 l tank at a pH of 7.3 at 60°C for about 7 hours, a hydrolyzate that has an NPN of 60% is obtained.

Example 2

The procedure is performed in a manner that is similar to the one that is described in Example 1, except for the fact that during the three separate tests, the useful volume of the tank is varied so that the NPN that is obtained after the passage of the substrate into the tank is respectively 15, 35 and 45%.

At the outlet of the tube, hydrolyzates that have the respective NPN of 59, 63 and 66% are thus obtained.

For comparison, under the same batch conditions in the tank, namely at pH 7.3 at 60°C with a substrate with 20% dry material and an amount of enzyme that has an activity of 3 AU per 100 g of dry material of the substrate, an NPN of 60% is obtained in about 7 hours.

In other words, with this process, an NPN is obtained continuously that is higher than the one that would be obtained intermittently as soon as the substrate has an NPN that is higher than about 35% at the inlet of the tube.

Example 3

The procedure is performed in a manner that is similar to the one that is described in Example 1, except for the fact that a pH of 7.8 and a temperature of 55°C instead of a pH of 7.3 and a temperature of 60°C are maintained in the tank.

At the outlet of the tube, a hydrolyzate that has an NPN of 70% is obtained.

Example 4

This process is implemented with a device of a type that is similar to the one that is described in reference to Figure 2.

Used as substrate is a concentrate of lactoserum proteins that have a content of dry material of 33% including 7.5% proteins.

Used as enzyme is a bacterial alkaline protease produced by Bacillus licheniformis and marketed by the Novo Company under the name "alcalase 2.4 L," which has an activity of 2.4 AU/g. This enzyme is used at a rate of a total amount of 4-8.6% on protein, or 2.2-4.7 AU per 100 g of dry material of the substrate.

2N KOH is used as a reagent.

After having started up the process in a suitable manner, it is continued in steady state. The flow rate of substrate and the dimensions of tubes and the tank are determined such that the dwell times of the substrate or the hydrolyzate are respectively 5-10 minutes

in a preliminary mixing tube that is equipped with static mixer elements that precede the tank, 5-8 minutes in a heat denaturation tube that is equipped with static mixer elements that are connected in series between the preliminary mixing tube and the tank, 25-40 minutes in the tank (1st hydrolysis stage), 15-25 minutes in a first tube A that is equipped with static mixer elements according to the tank (tube A of the 2nd hydrolysis stage), 15-25 minutes in a second tube B that is equipped with static mixer elements (tube B of the 2nd hydrolysis stage), 0-100 minutes in a third tube C that is equipped with static mixer elements (tube C of the 2nd hydrolysis stage), 5-20 minutes in a deactivation tube that is equipped with static mixer elements that are connected in series behind tube C, and 5-15 minutes in a cooling tube that is equipped with static mixer elements.

Said total quantity of enzyme is distributed in four portions, namely a first portion of 5-15% of the total that is mixed with the substrate in the preliminary mixing tube, a second portion of 30-40% of the total that is mixed with the substrate in the tank, a third portion of 20-30% of the total that is mixed with the substrate in said tube A, and a further portion of 20-30% of the total that is mixed with the substrate in said tube B.

The pH of the substrate is adjusted to 7.3 as far as up to said tube B, from which the pH is allowed to vary.

The temperature is adjusted to 75°C in the preliminary mixing tube, 85°C in the heat denaturation tube, 70°C in the tank, 71°C in tube A and tube B, 80-105°C in the deactivation tube and 2-8°C in the cooling tube.

The thus produced hydrolyzate is collected after the cooling tube.

Example 5

This process is implemented with a device of a type that is similar to the one that is described in reference to Figure 3.

Used as substrate is a concentrate of lactoserum proteins having a dry material content of 28%, including 7% proteins.

Used as enzyme is alcalase 2.4 L at a rate of a total amount of 2-6% on protein, or 1.2-3.6 AU per 100 g of dry material of the substrate.

2N KOH is used as a reagent.

After having started up the process in a suitable manner, it is continued in steady state. The flow rate of substrate and the dimensions of tubes and tanks are determined such that the successive stages take place as follows.

In a preliminary mixing tube that is equipped with static mixer elements preceding a prehydrolysis tank, 33% of the total amount of enzyme is mixed with the substrate at pH 8.7 at 10°C.

A first portion of the first hydrolysis stage is carried out in said prehydrolysis tank for 15 minutes at 65°C.

In a heat denaturation tube that is equipped with static mixer elements that are connected in series between the prehydrolysis tank and a hydrolysis tank, it is heated to 92°C for 5 minutes, then it is cooled to 65°C.

In said hydrolysis tank, the remaining 66% of the total amount of enzyme is added, and a second portion of the first hydrolysis stage is carried out for 45 minutes at pH 7.4 at 65°C.

In three tubes that are equipped with static mixer elements and connected in series downstream from the tank, the second hydrolysis stage is carried out at 65°C for 195 minutes, namely for 65 minutes in each tube. The pH is adjusted to 7.4 at the inlet of each tube and then floats.

In a deactivation tube that is equipped with static mixer elements and that is connected in series after the three hydrolysis tubes, an enzyme auto-digestion is carried out at 87°C for 5 minutes.

In a device for heating by vapor injection that is connected in series after the deactivation tube, sterilization is carried out at 125°C for 1 minute.

The hydrolyzate is then collected after having been cooled.

Example 6

This process is implemented with a device that is similar to the one that is described in reference to Figure 1, in which the hydrolysis tank has a volume of 2.8 l and the hydrolysis tube that is equipped with static mixer elements has a volume of 11.6 l for a length of about 5 m.

Used as substrate is a concentrate of lactoserum proteins that have a dry material content of 33%, including 7.5% proteins.

Used as enzyme is alcalase 2.4 L at a rate of a total amount of 6.3% on protein, or 3.4 AU per 100 g of dry material of the substrate.

2N KOH is used as a reagent.

First, the tank is filled with substrate, enzyme is mixed therein, and the batch hydrolysis process is started at a pH of 7.3 at 70°C for 25 minutes.

The process is then continued in steady state at a flow rate such that the total dwell time of the hydrolyzate in the device is 240 minutes (47 minutes in the tank and 193 minutes in the tube). A temperature of 70°C and a pH of 7.3 are maintained in the tank. A temperature of 70°C is maintained in the tube, and the pH is allowed to vary there such that it spontaneously decreases from about 7.3 at the inlet to about 6.72 at the outlet.

Samples are taken and analyzed at the outlet of the tube at times of 0, 60, 120 and 180 minutes starting from 240 minutes after the beginning of the continuous process. These samples have the pH values and amine nitrogen contents indicated in Table 1 below where the corresponding amount of KOH used to keep the pH at 7.3 in the tank was also recorded.

Table I

Time (Minutes)	pH	Amine Nitrogen (%)	KOH (g/h)
0	6.71	0.26	124
60	6.72	0.25	123
120	6.73	0.26	125
180	6.71	0.26	125

The amounts of KOH indicated in g/h correspond to a mean consumption of 44.375 g per l of the tank for a dwell time of 47 minutes.

It is noted in this Table I that the characteristics exhibited by the hydrolyzate virtually do not vary, regardless of the time when the samples are taken and analyzed at the outlet of the tube. It is also verified by polyacrylamide gel zone electrophoresis

(SDS-Page method) that the advantageous peptide profile of these samples, for the most part small peptides, also remains remarkably constant.

For comparison, the same substrate is subjected to enzymatic batch hydrolysis with the same enzyme, in the same enzyme:substrate ratio, in a 2 l tank at 70°C with a pH that is kept at 7.3 for 47 minutes. After these first 47 minutes, the pH is allowed to vary. Samples are taken and analyzed at 47 minutes starting from the beginning of the hydrolysis, then at different times as far as up to and beyond 240 minutes. These samples have the pH values and the amine nitrogen contents that are indicated in Table II below.

Table II

Time (Minutes)	pH	Amine Nitrogen (%)
47	7.30	0.21
67	7.0	0.22
140	6.77	0.24
197	6.75	0.27
240	6.72	0.26
300	6.68	
360	6.65	

The amount of KOH used to keep the pH at 7.3 during the first 47 minutes is 45.5 g per l of the tank.

It is noted in this Table II that the characteristics that are exhibited by the hydrolyzate that is obtained intermittently in a tank vary quickly, also after the time of

240 minutes corresponding to the continuous dwell time in the device that is used in this Example 6.

This demonstrates one of the advantages of this process in which no changes in the product should be feared that are comparable to the one that is produced during the time that is necessary for the evacuation of the tank in a batch process.

Example 7

This process is implemented with a device that is similar to the one that is described in reference to Figure 1, in which the hydrolysis tank has a volume of 5 l and the hydrolysis tube that is equipped with static mixer elements has a volume of 9.6 l for a length of about 5 m.

Used as substrate is a concentrate of lactoserum proteins that have a dry material content of 33%, including 7.5% proteins.

Used as enzyme is alcalase 2.4 L, at a rate of a total amount of 8% on protein, or 4.4 AU per 100 g of dry material of the substrate. Of this 8%, 2% is used in the tank and 6% is added at the inlet of the tube.

2N KOH is used as a reagent.

During two separate tests, first the tank is filled with substrate, the enzyme is mixed therein, and the batch hydrolysis process is started at a pH of 7.3 at two different temperatures, 72.5 and 74°C, for 40 minutes.

Each process is then continued in steady state at a flow rate such that the total dwell time of the hydrolyzate in the device is 116 minutes (40 minutes in the tank and 76 minutes in the tube). Respective temperatures of 72.5°C and 74°C and a pH of 7.3 are

maintained in the tank for each of the two tests. A temperature of 72°C is maintained in the tube, and the pH is allowed to vary therein.

The thus obtained hydrolyzates, corresponding to temperatures of 72.5°C and 74°C in the tank, have respective NPN of 97.2% and 91.4% and required the use of respective amounts of 205 g/h and 198 g/h of KOH to keep the pH at 7.3 in the tank. It is also observed by polyacrylamide gel zone electrophoresis (SDS-Page method) that they have a relatively narrow peptide profile.

For comparison, the same substrate is subjected to an enzymatic hydrolysis with alcalase 2.4 L, at a rate of a total amount of 4% on protein, or 2.2 AU per 100 g of dry material of the substrate, in steady state in a 5 l tank at 70°C for 200 minutes at respective pH values of 6.4, 6.8, 7.3 and 7.8 during four separate tests.

The thus obtained hydrolyzates have NPN between 80 and 80% and required the use of respective amounts of KOH, in g/h, of 33.4, 50.1, 60.2 and 77.2 to maintain their pH at 6.4, 6.8, 7.3 and 7.8. They also have respective contents of amine nitrogen of 0.17, 0.20, 0.21 and 0.22%. In addition, it is observed by SDS-Page testing that they have a relatively broad peptide profile.

This demonstrates another advantage of this process thanks to which it is possible to obtain a product that has a high degree of hydrolysis and a relatively narrow peptide profile, by comparison with a product that is obtained by continuous enzymatic hydrolysis in a tank that has a lower degree of hydrolysis and a relatively broad peptide profile.

Example 8

This process is implemented with a device that is similar to the one that is described in reference to Figure 1, in which the hydrolysis tank has a volume of 2.8 l and the hydrolysis tube that is equipped with static mixer elements has a volume of 11.6 l for a length of about 5 m.

Used as substrate is a concentrate of lactoserum proteins that have a dry material content of 33%, including 7.5% proteins.

Used as enzyme is alcalase 2.4 L, at a rate of a total amount of 7% on protein, or 3.8 AU per 100 g of dry material of the substrate. Of this 7%, 2% is used in the tank and 5% is added at the inlet of the tube.

2N KOH is used as a reagent.

First, the tank is filled with substrate, enzyme is mixed therein, and the batch hydrolysis process is started with a pH of 7.8 at 70°C for 25 minutes.

The process is then continued in steady state at a flow rate such that the dwell time of the hydrolyzate is 45 minutes in the tank and 170 minutes in the hydrolysis tube, 215 minutes in all. A temperature of 70°C and a pH of 7.8 are maintained in the tank. A temperature of 70°C is maintained in the tube, and the pH is allowed to vary there, such that it spontaneously decreases from about 7.8 at the inlet to about 6.67 at the outlet.

In a deactivation tube that is equipped with static mixer elements that are connected in series at the outlet of the hydrolysis tube, the hydrolyzate is deactivated for 18 minutes at 90°C. In a cooling tube that is equipped with static mixer elements that are connected in series downstream from the deactivation tube, the hydrolyzate is cooled to ambient temperature.

At the outlet of the cooling tube, samples are taken and analyzed that correspond to times of 0, 60, 120, 180 and 240 minutes starting from the outlet of the hydrolysis tube from 215 minutes after the beginning of the continuous process. These samples have the pH values, amine nitrogen contents, lysine blockages and NPN that are indicated in Table III below where the corresponding amount of KOH that is used to keep the pH at 7.8 in the tank was also recorded.

Table III

Time (Minutes)	pH	Amine Nitrogen (%)	KOH (g/h)	Lysine Blockage	NPN (%)
0	6.67	0.26	125	16.3	95
60	6.68	0.25	124	16.2	96
120	6.67	0.26	128	16.3	94
180	6.67	0.27	124	16.2	95
240	6.67	0.26	127	16.1	96

It is noted in this Table III, as can also be seen in Table I of Example 6, that the characteristics that are exhibited by the hydrolyzate virtually do not vary, regardless of the time when samples are taken and analyzed at the outlet of the hydrolysis tube.

The peptide profile and the hypoallergenic properties of the product that is obtained under the conditions according to this example are also examined by subjecting them to HPLC, ELISA and serotonin-^{3}H tests whose results are exhibited below in Tables V, VI and VII.

For comparison, 160 kg of the same substrate is subjected to an enzymatic batch hydrolysis with the same enzyme, at a rate of a total amount of 7% on protein, in a tank, at 70°C. Of this 7% enzyme, 2% is used for a first portion of the hydrolysis at a pH of 7.8 for 45 minutes after which the pH is allowed to vary. After 60 minutes, 5% remaining enzyme is added, and the hydrolysis is continued at 70°C and at a pH that varies as far as up to 215 minutes and beyond.

20 kg of hydrolyzate is drawn off after 120 minutes starting from the beginning of the hydrolysis. The same is done after 150, 180, 200, 250, 300 and 360 minutes. In transit, a sample is taken for analysis after 215 minutes.

The hydrolyzates that correspond to various samplings and samples are immediately deactivated (in a heat exchanger, 18 minutes at 90°C), then cooled at ambient temperature (in a heat exchanger) and analyzed. They exhibit the pH values, amine nitrogen contents (% powder at 97% of dry material), lysine blocking and NPN indicated in Table IV below.

Table IV

Time (Minutes)	pH	Amine Nitrogen (%, Powder)	Lysine Blocking	NPN (%)
60	7.56			
120	6.97	0.60	15.3	
150	6.87	0.63	17.2	90
180	6.82	0.66	18.2	94
200	6.80	0.68	18.3	95

Time (Minutes)	pH	Amine Nitrogen (%, Powder)	Lysine Blocking	NPN (%)
215	6.80	0.68	18.9	95
250	6.79	0.69	19.4	96
300	6.77	0.71	19.5	96
360	6.76	0.74	19.6	97

It is noted in this Table IV, as was done in Table II of Example 6, that the characteristics exhibited by the hydrolyzate obtained intermittently in the tank quickly vary, also after the 215 minutes corresponding to the continuous dwell time in the device that is used in this Example 8.

This confirms one of the advantages of this process in which no changes in the product should be feared that are comparable to the one that is produced during the time that is necessary for the evacuation of the tank in a batch process.

The peptide profile and the hypoallergenic properties of the product that is obtained under the conditions according to the comparison example below, in a time of 215 minutes, are also examined by subjecting them to HPLC, ELISA and serotonin-^{3}H tests whose results are exhibited below in Tables V, VI and VII.

Analogous tests are carried out on the product that is obtained in a continuous tank under the conditions that correspond to pH 7.3, exhibited for comparison in Example 7, and they are also exhibited in Tables V, VI and VII below.

Table V

Peptide Profile (HPLC Test)					
Product According to	% of Peptides Included in the Domains Included Within the Molecular Weight Limits Expressed in kDalton				
	>14	14-6	6-3.5	3.5-1.0	< 1
Example 8	5	7	9	30	49
Comparison (Batch Tank)	4	7	10	31	48
(Continuous Tank)	21	13	9	24	33

The results of tests according to this Table V well illustrate the fact that a hydrolyzate that is obtained continuously by the process according to this invention can have a peptide profile that is at least as narrow and as centered on the small peptides as a hydrolyzate that is obtained for comparison in a batch tank, whereas the peptide profile that is exhibited by a hydrolyzate that is obtained for comparison in a continuous tank has a much broader peptide profile that is shifted toward the large peptides.

Table VI

ELISA Inhibition Test			
Produced According to	Residual Antigenicity Expressed in ug of Antigen per g of Protein		
	BLG	BSA	CAS
Example 8	53	20	150
Comparison (Batch Tank)	41	7	141
Comparison (Continuous Tank)	111	> 1000	319

Table VII

Serotonin-^{3}H Release Test	
Produced According to	Residual Antigenicity Expressed in ug of Equivalent BLG for the Release per g of Protein Equivalent
Example 8	20
Comparison (Batch Tank)	5
Comparison (Continuous Tank)	50

The results of the tests according to these Tables VI and VII illustrate the fact that a hydrolyzate that is obtained by the process according to this invention can be at least as

hypoallergenic as a hydrolyzate that is obtained for comparison in a batch tank, whereas a hydrolyzate that is obtained for comparison in a continuous tank is not hypoallergenic.

Example 9

This process is implemented in the manner that is described in Example 7 under the conditions that correspond to a temperature of 72.5°C in the tank. The tube is subdivided into nine segments. Samples are taken between two successive segments as the continuous hydrolysis proceeds after the batch start-up. Samples are taken at the outlet of the tube at the same rate as soon as the continuous hydrolysis period reaches the time corresponding to the dwell time of the product in the tube.

The amine nitrogen content of the samples is determined. Each of these contents is divided by the equilibrium content toward which the hydrolyzate tends. In a coordinate system, the quotients that are obtained are recorded on the ordinates and the quotients of the sampling times divided by the dwell time of the hydrolyzate in the device are plotted on the abscissas.

A sigmoid curve is obtained that intersects starting at abscissa 0.8 and that crosses the vertical line of abscissa 1.0 at two thirds of its maximum value and reaches its maximum value, in other words touching the horizontal line of ordinate 1.0 at the vertical line of abscissa 1.2.

For comparison, the same test is carried out, with the exception of the fact that an empty tube is used that further exhibits the same dimensions as the tube that is equipped with static mixer elements.

Samples are taken under the same conditions, the same quotients are derived, and the corresponding curve is plotted in the same way.

A sigmoid curve is obtained that intersects starting at abscissa 0.6, crossing the vertical line of abscissa 1.0 at half of its maximum value and reaching its maximum value, namely 1.0, only beyond the vertical line of abscissa 1.8.

This demonstrates still two other advantages of this process, namely, on the one hand, the quickness with which a stationary process can be reached, and, on the other hand, the homogeneity that this hydrolyzate exhibits at the outlet of this device.

Claims

1. Process for enzymatic hydrolysis of proteins, in which a protein substrate is subjected to hydrolysis with a proteolytic enzyme, comprising a first enzymatic hydrolysis stage in a tank while being stirred, and a second enzymatic hydrolysis stage in a tube.
2. Process for continuous enzymatic hydrolysis, according to claim 1, in which said second hydrolysis stage is carried out in a tube that is equipped with static mixer elements.
3. Process according to claim 1, in which said substrate is a protein-rich, alimentary raw material, in particular of flours or semolinas of grains or oleaginous oilcakes, yeasts or alimentary bacteria, animal skin or ground fish, or milk or milk derivatives, in the form of particles of aqueous suspension or aqueous suspension.

4. Process according to claim 1, in which said protein substrate is a lactose substrate that contains lactoserum proteins, in particular a mild cheese-dairy lactoserum or an acidic cheese-making lactoserum, as is or in demineralized form or delactosed form, in liquid form or in reconstituted form.
5. Process according to claim 1, in which the proteolytic enzyme is selected from the group that is formed by trypsin, chymotrypsin, pancreatin, bacterial proteases, fungal proteases and mixtures thereof.
6. Process according to claim 1, in which the proteolytic enzyme and the substrate are mixed at a rate of an amount of enzyme that has an activity of 0.1-12 AU per 100 g of dry material of the substrate.
7. Process according to claim 1, in which the first stage is carried out for 10-60 minutes while adjusting the pH and the temperature to values that are favorable to the activity of the enzyme, and the second stage is carried out for 1-8 hours by adjusting the temperature to a value that is equal to or greater than, in particular 0-10°C, the temperature of the first stage.
8. Device for the implementation of the process according to claim 1, comprising a hydrolysis tank with a stirring mechanism that is connected upstream to a substrate-metering device and to an enzyme-metering device and connected downstream to at least one hydrolysis tube.
9. Device according to claim 8, in which said hydrolysis tube is equipped with static mixer elements.

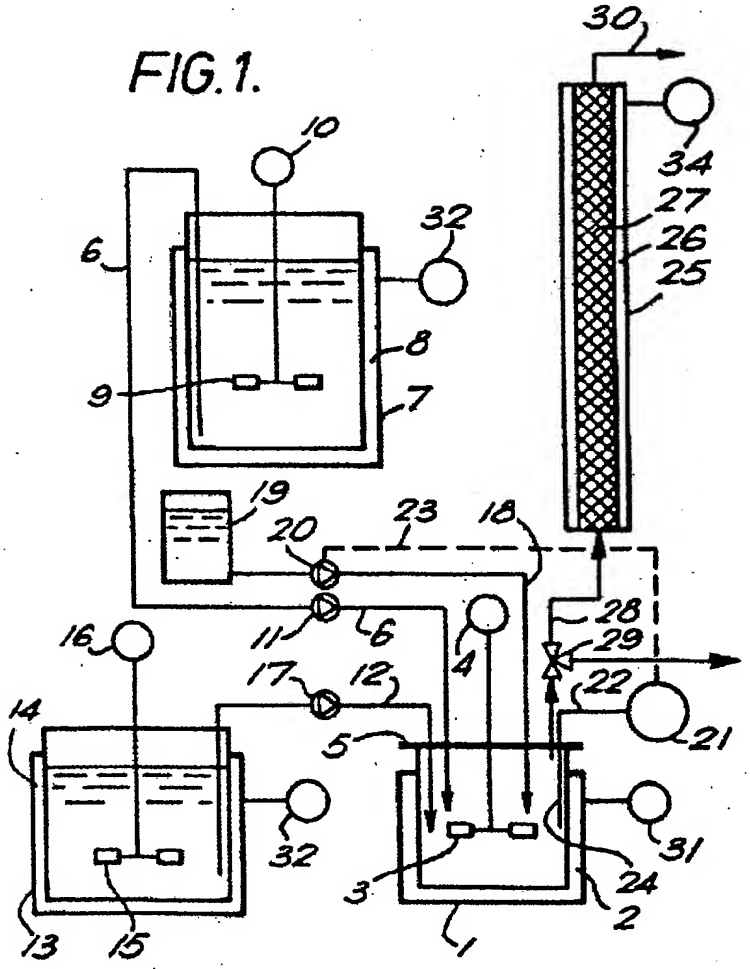
10. Device according to claim 9, in which said tube is placed vertically, whereby its lower end is connected to said tank and its upper end empties into a discharge hose.

11. Device according to claim 9, in which said substrate-metering devices and enzyme-metering devices each comprise a feed container that is connected to said hydrolysis tank.

12. Device according to claim 11, also comprising a reagent-metering device that comprises a feed container that is connected to the hydrolysis tank via a volumetric pump that is controlled by a pH-meter that is connected to the tank.

13. Device according to claim 12, comprising several hydrolysis tubes that are equipped with static mixer elements, connected in series downstream from the tank via connecting hoses connected upstream to the enzyme-metering device and the reagent-metering device.

FIG.1.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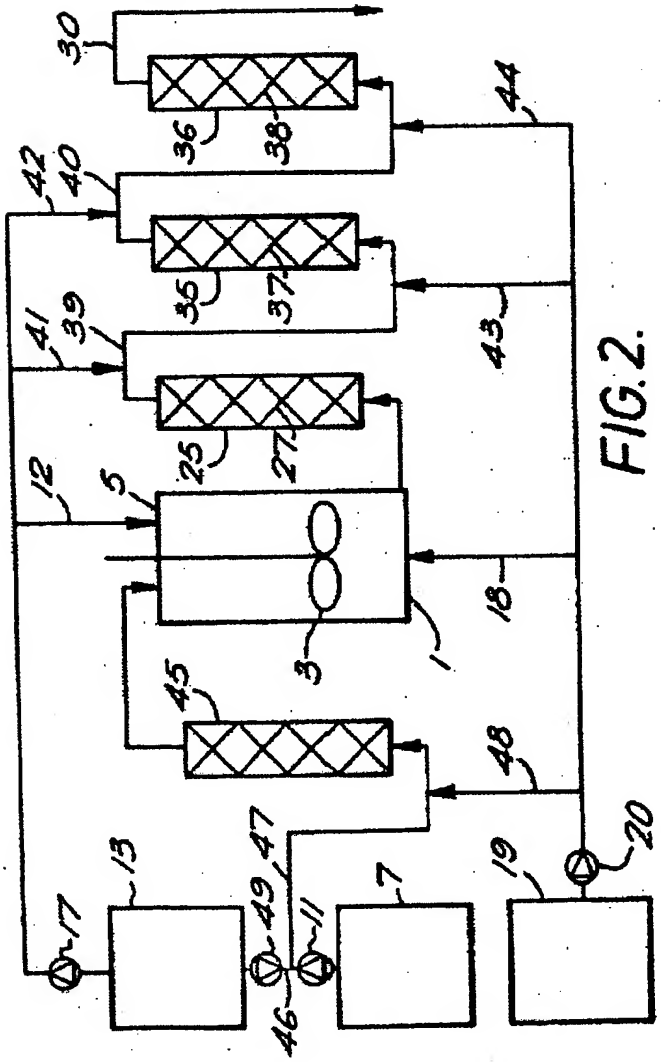

FIG.2.

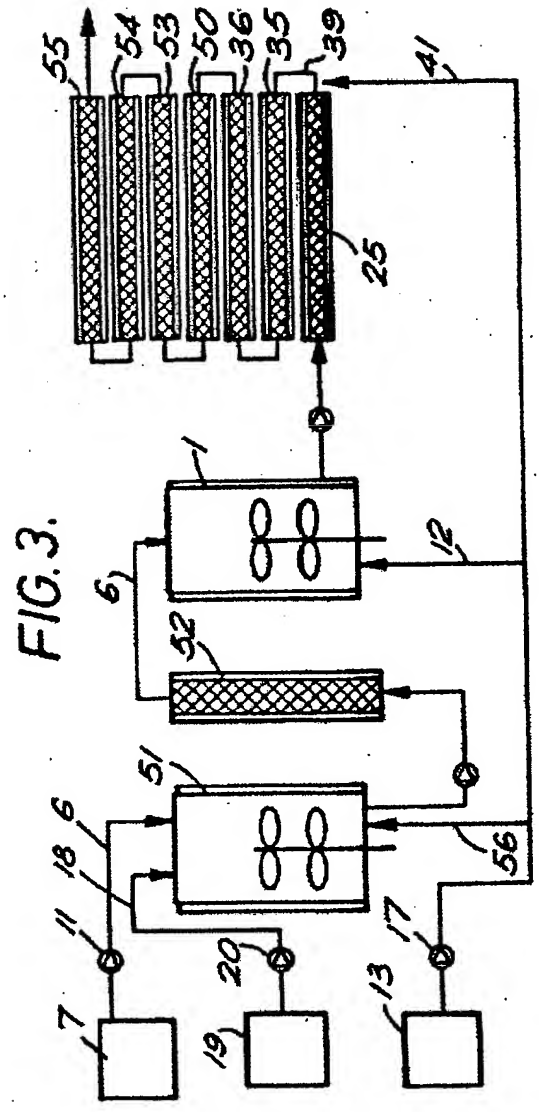

FIG.3.

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(71) Applicant: **SOCIETE DES PRODUITS NESTLE S.A.**
EPFL,
Institut de Génie Chimique CHB
CH-1015 Lausanne (CH)

Inventor: **Meister, Niklaus**
Möschbergweg 20
CH-3506 Grosshoechstetten (CH)

Inventor: **Renken, Albert**
EPFL,
Institut de Génie Chimique CHB
CH-1015 Lausanne (CH)

Inventor: **Wood, Robert Dustan**
Av. d'Echallens 63
CH-1004 Lausanne (CH)

Inventor: **Woupeyi, Alfred**
Rue Haldimand 24B
CH-1400 Yverdon (CH)

P.O. Box 353

CH-1800 Vevey (CH)

(72) Inventor: **Baensch, Johannes**

Rte du Port 28

CH-1009 Pully (CH)

Inventor: **Margot, Antoine**

(74) Agent: **Wavre, Claude-Alain**

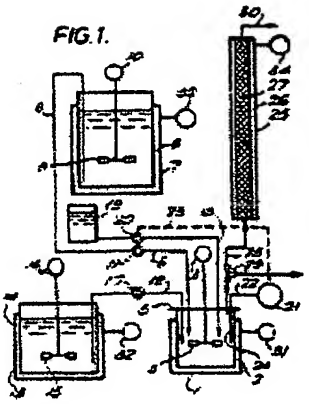
et al.

55, avenue Nestlé

CH-1800 Vevey (CH)

(54) **Enzymatic Hydrolysis of Proteins.**

(57) Process and device for enzymatic hydrolysis of proteins, in which are mixed a proteolytic enzyme and a protein substrate; a first stage of hydrolysis is carried out in a tank while being stirred, and a second stage of hydrolysis is carried out in a tube that is equipped with static mixer elements.



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PERTINENT DOCUMENTS			
Category	Citation of documents, with indication, if necessary, of pertinent passages	Related Claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	US-A-3 442 656 (D. A. MEADORS) * Column 2 – Column 3; Claim 1 * * Column 5 – Column 6; Figures 1, 2, 4 * ---	1-3, 5, 8-10, 13	C12P21/06 C12M1/06 C07K15/06 A23J3/34 C07K15/00
A	EP-A-0 286 838 (SOCIETE DES PRODUITS NESTLE S.A.) * Column 7; Claims 2, 3, 5, 9; Example 1* ---	1-3, 8, 9, 12	
A	US-A-4 464 509 (MARATHON OIL COMPANY) * Column 3 – Column 4; Claims 1, 7, 9; Figure 1 * ---	8-10	
A	EP-A-0 274 946 (LABORATOIRE ROGER BELLON S.A.) * Page 3; Claims 1, 2, 4; Example 1 * ---	1, 3-5	
A	DATABASE WPI Week 8815, Derwent Publications Ltd., London, GB; AN 88-099079 & DD-A-251 539 (VEB LEUNA-WERK ULBRIGHT) November 18, 1987 * Abstract* ---	1, 2, 8-10, 13	TECHNICAL SEARCH FIELDS (Int. Cl. 5) C12P C12M A23J C07K A23L

P, A	US-A-5 174 651 (GADDIS PETROLEUM CORPORATION) * Column 5; Figure 1 * ---	8-10	

This report was drawn up for all of the claims

Location of the Search	Date the Search was Completed	Examiner
THE HAGUE	September 26, 1994	Kanbier, D.

CATEGORY OF CITED DOCUMENTS

A: Technological background

P: Insert